Genetic Variation at the **IL10** Gene Locus Is Associated with Severity of Respiratory Syncytial Virus Bronchiolitis

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The intense airway inflammatory response associated with respiratory syncytial virus (RSV) infection may be an important determinant in the severity of the disease. Interleukin (IL)–10 is a key regulatory cytokine known to be secreted during this infection. We investigated the role that IL-10 plays in RSV disease by studying the effects that variation in the **IL10** gene has on the outcome of the disease. Eight single nucleotide polymorphisms (SNPs) spanning the **IL10** gene were selected, and haplotypes were constructed. SNPs that efficiently tagged these haplotypes were then typed in 580 infants with severe RSV bronchiolitis and in 580 control subjects. None of the SNPs or haplotypes was associated with RSV bronchiolitis. In a subgroup analysis, 2 SNPs (**IL10*/H11002*/H11002*/1117 and **IL10*/H11002*/H11002*/3585) were associated (odds ratio, 1.7; \( P = .004 \)) with the need for mechanical ventilation. These data are consistent with the theory that **IL10** plays a role in the severity of RSV infection in infants.

Bronchiolitis caused by respiratory syncytial virus (RSV) is the most common reason that infants in the developed world are admitted to the hospital [1], and it is an important contributor to morbidity and mortality in infants worldwide. By the end of their second winter, nearly all infants will have been infected with RSV [2]. The majority of infected infants will have coryzal symptoms with or without a cough. A small proportion (1%–2%) develop severe lower respiratory tract disease that necessitates supportive care in the hospital. The host response to RSV appears to be an important determinant of the severity of the disease.

It has been suggested on the basis of mouse models of RSV infection that a Th2-weighted immune response favors the development of severe bronchiolitis [3]. Some studies of the levels of Th1 and Th2 cytokines in nasal secretions and blood from infants with severe RSV disease have found evidence to support this model, whereas others have not.

Interleukin (IL)–10 is an obvious candidate for investigation. IL-10 is a critical antiinflammatory cytokine that is known to suppress Th1-like immune responses and promote Th2 responses. IL-10 is produced by Th2 cells, B cells, monocytes, and macrophages, and it antagonizes the production of proinflammatory cytokines, including tumor necrosis factor, IL-1, IL-6, IL-18, leukemia inhibitory factor, granulocyte-macrophage colony-stimulating factor, and IL-12 [4]. IL-10 also down-regulates the expression of major histocompatibility complex class II, intercellular adhesion molecule 1, CD80, and CD86, as well as inhibits the production of prostaglandin E₂ in monocytes [5]. IL-10 thus inactivates the cell-mediated immune response through a combination of cytokine, chemokine, and antigen presentation inhibition. Conversely, IL-10 enhances humoral immunity by promoting the proliferation of B cells and T cells.

Levels of IL-10 in nasal secretions increase markedly during RSV infection. Because IL-10 is not secreted by cells in the respiratory system, the source of the increased IL-10 is likely to be T cells or mast cells involved.
in the host immune response. Very few studies have correlated IL-10 levels with the severity of RSV disease in infants. Legg et al. found that the IL-10:IL-12 ratio in nasal secretions was higher in infants with acute RSV bronchiolitis than in those with coryzal symptoms only [6]. Bont et al. found no difference in the mean levels of IL-10 between infants with RSV who required mechanical ventilation and those who had milder disease [7]. Although measurements of cytokine concentrations during acute RSV infection provide invaluable information, they are difficult to obtain. There are inevitable variations in the ages of the infants, the definition of the phenotype, the timing of the collection of each sample in relation to the onset of the infection, the nature of the samples collected, the selection of the control subjects, and the methods used to perform the assays. Finally, even when a clear correlation exists between cytokine levels and the severity of disease, it can be difficult to be certain whether the high levels of cytokines are a consequence or a cause of the severe phenotype. Genetic association studies provide a complementary approach that avoids many of these difficulties. If functional genetic variations are associated with disease, then it can be reasonably stated that the genes whose function is altered by the variation are directly involved in the pathogenesis of the disease. We and others have successfully used this approach to study a number of inflammatory mediators in RSV disease [8–12]. Here we used genetic association methods to investigate whether variation at the IL10 gene locus affects the severity of disease in infants with RSV bronchiolitis.

**SUBJECTS AND METHODS**

**Subjects.** Infants with RSV-positive (by immunofluorescence or culture) bronchiolitis were identified at 10 hospitals in southeastern England. To be included in the study, patients had to be <12 months of age and had to have RSV-positive bronchiolitis characterized by tachypnea, retractions, and bilateral crackles (having wheeze alone was not considered to be sufficient for inclusion in the study). Infants were included in the study only if their disease was considered to be severe enough to necessitate gavage feeding, intravenous fluids, or supplemental oxygen. DNA samples were collected from 580 case patients. Seventy-nine

### Table 1. Characteristics of the study population.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Case patients (n = 580)</th>
<th>Case patients requiring mechanical ventilation (n = 79)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean, weeks</td>
<td>16.4 ± 8.8</td>
<td>8.8 ± 8.8a</td>
</tr>
<tr>
<td>Male</td>
<td>302 (53)</td>
<td>48 (61)</td>
</tr>
<tr>
<td>Length of hospital stay, mean (range), days</td>
<td>6.04 (1–34)</td>
<td>11.4 (3–26)</td>
</tr>
<tr>
<td>Supplemental oxygen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>456 (80)</td>
<td>79 (100)</td>
</tr>
<tr>
<td>Length of therapy, mean (range), days</td>
<td>4.4 (1–30)</td>
<td>9.1 (2–22)</td>
</tr>
<tr>
<td>Gavage feeding/intravenous fluids</td>
<td>404 (70)</td>
<td>79 (100)</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>79 (14)</td>
<td>79 (100)</td>
</tr>
<tr>
<td>Preterm birth (gestation &lt;37 weeks)</td>
<td>116 (20)</td>
<td>28 (35) a</td>
</tr>
<tr>
<td>Preexisting heart/lung disease</td>
<td>58 (10)</td>
<td>16 (20) a</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%) of subjects, unless indicated otherwise. a Significantly different between all case patients and case patients requiring mechanical ventilation (P <0.05).
infants required mechanical ventilation, and this requirement was used as an objective marker of severity. The clinical characteristics of the case patients are shown in table 1. DNA samples were also collected from 580 infants born consecutively at the John Radcliffe Hospital in Oxford during 1999–2000. All subjects included in the study were of white European descent. Informed consent was obtained from the parents of all participants, and the study was approved by the East Anglian Multi-Centre Ethics Committee.

**DNA collection.** Mouth swabs were used to collect samples from the case patients and their parents. Cord blood samples were collected from the control subjects. The DNA was extracted, preamplified, and stored as described elsewhere [8].

**SNP genotyping.** SNP genotyping was performed by use of primer extension mass spectrometry and MassARRAY software (Sequenom) [13]. One SNP, rs1800890, proved refractory to this method and was genotyped by restriction fragment–length polymorphism analysis. All primer sequences and assay conditions are available on request.

**Analysis.** Haplotypes were constructed from the family data and from data from unrelated individuals by use of PHASE software (version 2) [14]. Comparisons between case patients and control subjects were made by use of simple 2 × 3 tables with calculated odds ratios. Subgroup analysis was performed by taking into account the marker of severity (need for mechanical ventilation) and known risk factors (preterm birth [gestation <37 weeks] and preexisting heart or lung disease). Binary logistic regression was used to determine if there were significant gene–gene or gene-environment interactions.

**RESULTS**

**Selection of informative SNPs.** We began by investigating 8 SNPs in an 8-kb region spanning the *IL10* gene, including 3 kb of the 5′ flanking region. All had been observed at ≥0.10 frequency and were known to occur in white Europeans. They included *IL10* −1117, *IL10* −854, and *IL10* −627 (previously referred to in the literature as *IL10* −1082, *IL10* −819, and *IL10* −592, respectively), all of which have been associated with the altered transcriptional regulation of *IL10* [15–18]. They were genotyped in 32 white family trios (mother/father/child) from the United Kingdom, and the haplotype structure was determined by first identifying pedigrees in which the phase was unambiguous and then using the PHASE algorithm to estimate the remaining haplotypes [19]. The frequencies of SNP alleles and of haplotypes were then calculated for the 128 parental chromosomes. Details of the SNPs examined and of their minor allele frequencies in this study population, which ranged from 0.16 to 0.50, are shown in table 2. Using the ENTROPY algorithm [19], we identified 4 SNPs that captured >80% of the observed haplotype diversity; these are identified in table 2, and the haplotypes are shown in table 3. Interestingly, *IL10* −854 and *IL10* −627 occur at the same frequency and are in complete phase and linkage disequilibrium (LD). The inclusion of both markers in the construction of haplotypes did not add information that was not present when either marker was used alone. Pairwise LD statistics (*D*′ and *R*′) for all 8 SNPs are presented in tables 4 and 5.

**Case-control analysis of the association with RSV bronchiolitis.** We analyzed the 4 tagging SNPs in 580 case patients with severe RSV bronchiolitis and in 580 control subjects. All markers were typed with an efficiency of ≥95%. The distribution of genotypes from all 4 markers conformed to Hardy-Weinberg expectations in the case patients and in the control subjects. None of the 4 tagging SNPs showed a significant association with RSV bronchiolitis. Five common haplotypes were identified in the case patients and the control subjects. There were no significant differences in the distribution of the haplotypes between the 2 groups.

We then analyzed the subgroup of 79 infants with severe RSV bronchiolitis who required mechanical ventilation. In this subgroup, there was a significant association with the *IL10* −1117 and the *IL10* −3585 SNPs (table 6). Comparisons of haplotype frequencies between case patients and control subjects identified a similar effect with haplotypes carrying the *IL10* −1117G allele. The associations detected were not stronger than that identified with the *IL10* −1117G allele alone. Infants with severe RSV bronchiolitis who required mechanical ventilation were younger, were more likely to have been born prematurely (gestation <37 weeks), and were more likely to have preexisting heart or lung disease than were infants who did not require mechanical ventilation (table 1). To assess whether the association between *IL10* SNPs and severe disease was independent of these other risk factors, a logistic regression model was used. The effect of *IL10* −3585 was weaker than that of *IL10* −1117, and that effect was undetectable when both SNPs were included in the model. This can be accounted for by the relatively high LD between the 2 SNPs (*D*′ = 1.0; *R*′ = 0.8). *IL10* −1117 re-

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**Table 3. Haplotypes derived from 8 single nucleotide polymorphisms (SNPs) spanning the *IL10* gene in 32 white families from the United Kingdom.**

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases.*

**Table 4. Linkage disequilibrium statistic *D*′ for all pairwise comparisons in the 8 single nucleotide polymorphisms (SNPs) spanning the *IL10* gene in 32 white families from the United Kingdom.**

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases.*
enough to necessitate mechanical ventilation is a reflection of the high LD between these 2 markers ($D' = 1.0$; $R^2 = 0.8$). Because haplotype analysis did not improve the strength of the association we observed when we used $IL10 - 1117$ alone, this suggests that the effect is due either to direct functional effects of this SNP or to an unobserved genetic variant in strong LD with $IL10 - 1117$.

Two previous genetic association studies of RSV bronchiolitis have used $IL10$ SNPs. Hoebee et al. [10] studied the $IL10 - 627$ SNP (which they referred to as $IL10 - 592$) and found no association with RSV bronchiolitis in 207 infants with the disease who were admitted to the hospital, which is consistent with our results. In a subgroup analysis, they found an excess of the $IL10 - 627A$ allele in infants ≤6 months old. We performed a similar analysis and were unable to replicate this result. Gentile et al. [11] studied 77 infants with RSV bronchiolitis and used the $IL10 - 1117$ and $IL10 - 627$ SNPs (which they referred to as $IL10 - 1082$ and $IL10 - 592$, respectively) to assign individuals to categories according to high-, intermediate-, or low-level production of IL-10. They found an association between the development of RSV pneumonia and low-level production of IL-10. They did not find an association with admission to the intensive care unit (ICU), although only 6 infants developed pneumonia and 15 required ICU care. It is not clear whether admission to the ICU necessarily entailed the need for mechanical ventilation. The authors did not publish the effects of individual SNPs, and it is hard to make a direct comparison with the results of our study. Lack of reproducibility is not unusual in genetic association studies and may reflect differences in both the phenotypes and the populations studied. It can also arise for stochastic reasons, particularly when sample sizes are relatively small and the observed effects are weak. If our data are taken together with data in previous studies, it is probably reasonable to say that, in infants with RSV bronchiolitis, variation in the $IL10$ gene does not influence the risk of being admitted to the hospital. However, variations in $IL10$ may be important in subgroups of infants, and our data suggest that it influences the risk of requiring mechanical ventilation.

Table 5. Linkage disequilibrium statistic $R^2$ for all pairwise comparisons of the 8 single nucleotide polymorphisms (SNPs) spanning the $IL10$ gene in 32 white families from the United Kingdom.

The table is available in its entirety in the online edition of the Journal of Infectious Diseases.

remains a significant predictor of severe disease when the other risk factors are taken into account (table 7).

**DISCUSSION**

In the present study, we used 8 SNPs spanning the $IL10$ gene to describe the genetic variability at this locus. By constructing haplotypes, we were able to select 4 tagging SNPs that identified >80% of the haplotype variability. Two of these SNPs, $IL10 - 1117G$ and $IL10 - 3585A$, showed significant association with RSV bronchiolitis severe enough to necessitate mechanical ventilation.

It has been estimated that ~70% of the interindividual variation in the production of IL-10 is genetically determined [20], and, because of the critical role that IL-10 plays in the human immune response, $IL10$ has been a popular choice for genetic association studies of inflammatory disease. Many studies have utilized $IL10$ promoter polymorphisms—in particular, $IL10 - 1117$—since Turner et al. [21] proposed that this transition resulted in a functional change in the production of IL-10 in vitro. Although it has been postulated that $IL10 - 1117$ contributes to differences in the innate production of IL-10, it is difficult to localize functional effects by genetic association methods, because of the presence of LD between polymorphic variants [22]. Significantly high levels of LD are present across the $IL10$ region [23], particularly in European populations [22, 24]. Thus, effects measured by using $IL10 - 1117$ may be due to functional variation at more distant elements. Our observation that both the $IL10 - 1117$ and $IL10 - 3585$ SNPs were associated with RSV bronchiolitis severe enough to necessitate mechanical ventilation is a reflection of

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype comparison, $P$</th>
<th>Allele 2</th>
<th>Genotype 2/2</th>
<th>Genotype 1/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$IL10 - 1117$</td>
<td>.01</td>
<td>1.68 (0.004)</td>
<td>2.06 (0.006)</td>
<td>0.54 (NS)</td>
</tr>
<tr>
<td>$IL10 - 3585$</td>
<td>.03</td>
<td>1.58 (0.01)</td>
<td>1.91 (0.03)</td>
<td>0.58 (NS)</td>
</tr>
</tbody>
</table>

**NOTE.** For $IL10 - 1117$, allele 1 = A and allele 2 = G; for $IL10 - 3585$, allele 1 = T and allele 2 = A. The OR for allele 2 is the odds of case patients having allele 2 compared with the odds of control subjects having allele 2; the OR for genotype 2/2 (allele 2 and allele 2) is the odds of case patients having genotype 2/2 compared with the odds of control subjects having genotype 2/2; the OR for genotype 1/1 (allele 1 and allele 1) is the odds of case patients having genotype 1/1 compared with the odds of control subjects having genotype 1/1. NS, not significant.
and that it existed on a haplotype associated with significantly decreased transcription of \textit{IL10} in vitro. \textit{IL10} \textsuperscript{−1117G}, \textit{IL10} \textsuperscript{−854C}, and \textit{IL10} \textsuperscript{−627C} form the “GCC” haplotype, which has previously been associated with increased secretion of IL-10 [15, 16]. This result conflicts with the data on \textit{IL10} \textsuperscript{−3585A}, because \textit{IL10} \textsuperscript{−1117G} exists most commonly on an extended haplotype with \textit{IL10} \textsuperscript{−3585A} [17, 18]. Inconsistent results from in vitro functional studies may arise because of differences in experimental design, including the use of different stimuli and different tissues [18]. It is therefore difficult to speculate whether increased or decreased production of IL-10 is associated with severity of disease in RSV bronchiolitis. Nevertheless, variation in the production of IL-10 does appear to be heritable, and SNPs in the promoter region are likely to be good markers of that variation. The data presented in this study are consistent with the theory that IL-10 plays a role in determining the severity of RSV disease. Further work is needed to localize the functional element responsible and to determine the effect that this element has on the production of IL-10 in infants infected with RSV.

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References